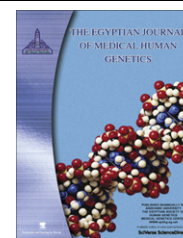




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ORIGINAL ARTICLE

Serine protease (TPS): A diagnostic and prognostic marker in pediatric patients with acute non-lymphoblastic leukemia

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Abstract The Serine protease, TPS (tryptase), is a specific marker for mast cells and mast cell-associated disorders. However, substantial amounts of TPS are also expressed in neoplastic myeloid, non-mast cell lineage. The aim of this study is determination and quantitation of TPS expression in patients with acute non-lymphoblastic leukemia (ANLL); to evaluate its prognostic value and its relevance as a genetic marker for detection of minimal residual blast cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect levels of TPS from 30 newly diagnosed ANLL children and 10 normal children served as controls. TPS levels for positive cases were reevaluated after induction chemotherapy; after onset of relapse or by the end of the study. Our results showed that the gene transcripts were detected in 56.7% of patients but were not expressed by normal controls. The highest frequency of TPS was recorded in patients with M4 showing significantly higher levels compared to other FAB (French–American–British Classification) subtypes. TPS levels were directly correlated to TLC, absolute blast counts in peripheral blood, levels of CD34 and CD117. After induction chemotherapy, levels of TPS decreased significantly in those who achieved complete remission while it increased significantly in relapsed patients,

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with a risk estimate of relapse six times higher in positive cases than TPS negative patients. 84.6% of the patients negative for TPS achieved complete remission with better disease free survival. In conclusion, TPS is expressed in a group of patients with ANLL thus serves as a disease related marker; it could be used as a prognostic indicator in evaluating remission status and early relapse as its elevated levels are associated with high risk of relapse; again, it is useful in predicting disease outcome.

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1. Introduction

Acute non-lymphoblastic leukemia (ANLL) is characterized by clonal proliferation of immature myeloid cells without significant differentiation [1]. Despite maturation arrest, blast cells in ANLL are often capable of expressing lineage-restricted differentiation antigens [2]. The prognosis and clinical picture in ANLL varies, depending on deregulated genes, cell type(s) involved and the specific biological properties of the clone(s) [3].

Tryptase are lineage-associated serine proteases primarily expressed in mast cells, and less abundantly in blood basophils [4]. The genes of TPS (a and b) cluster on the short arm of human chromosome 16 [5] most of the a-TPS product is enzymatically inactive (a-proTPS) and is released spontaneously by mast cells [6], while most of the b-TPS product is enzymatically active tetramers stored in cytoplasmic granules and released during degranulation [7]. Under physiologic conditions other myeloid cells are virtually TPS negative [8]. Later, it was found that myeloblasts in a group of patients with ANLL produce significant amounts of TPS [9] which induce a number of growth regulators involved in the survival and proliferation of blast cells [10].

In the present study, we try to detect and to quantitate the TPS expression in newly diagnosed Egyptian children with ANLL, to investigate its usefulness as a tool for diagnosis and prognosis of ANLL and as a marker of the disease outcome.

2. Subjects and methods

2.1. Subjects

This study was carried out on 30 newly diagnosed ANLL patients presenting to Hematology Oncology Clinic of Pediatrics Department, Ain Shams University Hospitals during the period between May 2008 and March 2010. Their mean age was 7.1 ± 4.3 years with a male:female ratio of (1.3:1). Ten age and sex matched normal subjects were included as controls. Parents of all participants signed an informed consent before starting the study. Exclusion criteria included patients with drug-related ANLL, biphenotypic leukemia, those having allergic reactions, helminthic infestations and renal disease. The diagnosis of ANLL was based according to the standard morphological and immunophenotypic criteria proposed by the French-American-British (FAB) cooperative study group (from M0 to M7).

According to the TPS gene expression, the patient group was subdivided into either negative or positive cases. Positive cases at presentation were re-evaluated for TPS mRNA after induction chemotherapy; if relapse occur during the follow up period or by the end of the study.

All patients except those with M3 received the standard induction protocol for ANLL, two cycles (3 + 7) consisting of daunorubicin 45 mg/m^2 for 3 days and cytosine arabinoside $100 \text{ mg/m}^2/12 \text{ h}$ for 7 days, followed by three cycles of consolidation cycle (A) consists of vepesid and adriamycin, cycle (B) consists of high dose cytosine arabinoside ($1 \text{ gm/m}^2/12 \text{ h}$) for 5 days followed by cycle (A) again. Patients with M3 received all-trans retinoic acid. Patients were followed up for a period of 12–24 months.

2.2. Methods

2.2.1. Sampling

Three milliliter anticoagulated EDTA blood were collected from all subjects, 2 ml for CBC and Leishman stained smears and 1 ml for DNA extraction and detection of TPS mRNA. One milliliter of bone marrow (BM) or peripheral blood was used for immunophenotyping (IPT) from patients only.

2.2.2. Baseline assessment

All patients were subjected to: full history taking, thorough clinical examination and laboratory investigations including complete blood picture and blood smears using Coulter Counter Gen-S (Coulter Electronics Corporation, Hielach, Florida, USA).

2.2.3. Bone marrow (BM)

Aspiration for detection of infiltration by $\geq 20\%$ blast cells of all nucleated BM cells and immunophenotypic analysis performed by multicolor direct immunofluorescence cytometry using Coulter EPICS XL flow cytometer (Florida, USA). The panel of MoAbs used for each sample includes: CD34, HLA-DR, CD13, CD33, CD14, CD15, CD7, and CD117.

2.2.4. Detection of TPS messenger RNA (mRNA)

Using reverse transcriptase-polymerase chain reaction (RT-PCR) [11] for patients and controls. Isolation of peripheral blood mononuclear cells (MNC) was performed by density gradient centrifugation using Ficoll Hypaque 1077 (Sigma USA) at $1200g$ for 30 min at 40°C , the interface cells were removed, washed twice with 25 ml sterile PBS (pH:7.3). Isolation of peripheral blood mononuclear cells (MNC) was performed by density gradient centrifugation using Ficoll Hypaque 1077 (Sigma, USA) at $1200g$ for 30 min at 40°C , the interface cells were removed, washed twice with 25 ml sterile PBS (pH:7.3), pelleted and re-suspended in 1 ml PBS. The cells were re-centrifuged at $1200g$ for 2 min then kept at -800°C until RNA extraction.

Total nuclear RNA was extracted using RNA Extraction kit (Qiagen). The isolated RNA was re-suspended in RNase-free water and stored at -80°C until assay. The RNA concentration was assessed by absorbance reading at 260 nm with UV spectrophotometry (Beckman; DU series 650, INC, USA).

Reverse transcription reaction for cDNA synthesis was carried out in 20 μ l reaction mixture by using first strand cDNA synthesis kit (Promega USA) according to manufacturer instruction.

PCR amplification was performed using the selected TPS primer pairs (59 primer: 59-GAGGCCCCCAGGAGCAAGTG-39; 39 primer: 59-ACATCGC-CCCAGCCAGTGAC-39), and β -actin gene, a house keeping gene used as an internal control (59 primer: 59-AGGCCGGC-TTCGCGGGCGAC-39; 39 primer: 59-CTCGGGAGCCACCAGCAGCTC-39).

A 5 μ l of first strand cDNA was used as a template for the PCR reaction. Each reaction mixture consisted of 0.5 mM of each primer, 10 \times Taq Buffer, 2.5 mM dNTP mix, 0.5 U of Taq polymerase and nuclease free water to a final volume of 50 μ l.

Thermocycling, using PTC 200 (MJ Research, Inc., Boston, Mass.), consisted of 32 PCR cycles (94 $^{\circ}$ C for 1 min, 63 $^{\circ}$ C for 1 min, and 72 $^{\circ}$ C for 90 s; initial denaturation step at 95 $^{\circ}$ C for 1 min). After amplification, PCR products were subjected to gel electrophoresis and visualized by ethidium bromide staining. Detection of a band at 383 bp indicated TPS gene amplification while β -actin gene was visualized at 200 bp (Fig 1). They were semiquantitated by the UVP Gel-Document System (GDS 8000). No template cDNA samples were used as negative control.

To normalize the difference in RNA degradation for individual samples and in RNA loading for RT-PCR, the values of TPS gene expression were divided by β -actin gene values in each sample and were defined as TPS gene expression levels.

2.3. Statistical analysis

Data were analyzed using the statistical software SPSS (version 10), results were expressed as mean values \pm standard error of mean (SED). Student's *t* test was used for comparison between two sets of parametric data. Chi square (χ^2) test was performed to compare between non-parametric data. Pearson's correlation (*r*) test was used for correlations between different parameters. Event free survival and overall survival were measured from start of disease until disease relapse/death or till the end of the study over a period of 12–24 months. Survival analysis was estimated using Kalpan–Meier curve. *p*-Value <0.05 was considered significant.

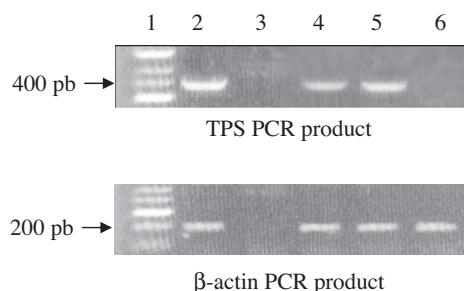


Figure 1 Electrophoretic separation of PCR amplified products. Lane 1: molecular DNA marker. Lanes 2, 4, and 5: cases showing positive TPS gene transcript and β -actin. Lane 3: negative control (blank). Lane 6: case showing β -actin only.

3. Results

The results of this study are summarized in Tables 1–4 and Figs. 1 and 2.

TPS together with β -actin genes expression were detected in 17 cases (56.7%) while all controls were negative for TPS showing β -actin gene only (Fig. 1). The TPS transcripts were particularly expressed with FAB groups M0 (4 of 5 patients), M1 (3 of 7), M2 (4 of 8), M3 (1 of 2) and was found in all M4 patients. But it was not expressed by M5 nor M6 subtypes. On comparing the hematological parameters in both groups, total leukocytic counts (TLC) were significantly higher while hemoglobin levels and platelet counts were significantly lower in patients expressing TPS ($p < 0.05$; Table 1). The TPS mRNA levels were higher in M4 patients ($12.1 \pm 3.4 \times 10^{-2}$) compared to those detected in other FAB subtypes ($4.6 \pm 1.3 \times 10^{-2}$) with a statistical significant difference ($t = 2.39$; $p = 0.03$; Table 2). Correlative studies showed that TPS levels were positively correlated with TLC; absolute blast counts in peripheral blood (PB), CD34, and CD117 levels (Table 3).

After the first induction chemotherapy cycle, the gene expression for the positive cases was reassessed and revealed low values of TPS in 8 patients ($0.71 \pm 0.26 \times 10^{-2}$) while the other nine showed elevated levels ($13.3 \pm 3.04 \times 10^{-2}$) with a statistical significant difference ($t = 4.15$; $p = 0.004$); 5 of these 9 patients showed incomplete remission by BM examination, while the other four were morphologically in complete remission (BM blasts <5%), yet. TPS mRNA remained highly expressed. During the follow up period, all of the nine cases relapsed.

84.6% of TPS negative patients showed complete remission (CR) which is statistically better outcome than positive cases (Table 4). Moreover, negative cases for TPS had longer overall survival (9.8 ± 0.58 months) than positive cases (6.9 ± 0.52 months) with a statistical significant difference ($t = 3.73$; $p = 0.001$). Survival analysis was estimated using Kalpan–Meier curve (Fig. 2). The risk estimate for relapse in patients expressing TPS was six times higher than those who were not expressing TPS with a 95% confidence interval CI (5.46–8.0).

5. Discussion

Despite progress in understanding molecular biology of ANLL, its treatment remains challenging. Advances in understanding the pathophysiology of ANLL may lead to improvements in survival of these patients [12], thus identification of new molecular markers for sub-classification and risk assessment is critical. Recent data suggested that the serine protease, tryptase, is expressed in a group of patients with ANLL and that its level reflects the burden of leukemic cells [3].

In the present study, TPS transcripts were expressed by 56.7% of patients but they were not expressed totally in controls. Also, in previous studies [5,10] the expression of TPS was confined to ANLL patients but not in controls nor in ALL groups. This proves that TPS is produced by leukemic myeloid blasts, which reflects inherent ability to produce TPS, and its specificity to myeloid cells. On the other hands, other investigators [8] showed that TPS was expressed in only 39% of cases. This discrepancy was due to the higher sensitivity of the PCR used in this report compared to other methods

Table 1 Comparative study of TPS gene expression in patients group and other hematological parameters at presentation.

Parameters	TPS gene expression		<i>t</i> -Test or χ^2	<i>p</i>
	Negative (13) $\bar{X} \pm \text{SED}/n$ (%)	Positive (17) $\bar{X} \pm \text{SED}/n$ (%)		
Age (years)	6.41 \pm 0.95	7.84 \pm 1.2	0.91	0.36
Gender male 17	10 (58.8)	7 (53.8)		
female 13	7 (41.2)	6 (46.2)	10.74	0.538
TLC ($\times 10^9/\text{l}$)	7.8 \pm 2.3	34.9 \pm 11.1	2.38	0.02*
Hemoglobin (g/dl)	7.9 \pm 0.5	5.6 \pm 0.5	3.02	0.006*
Platelets ($\times 10^3/\text{ml}$)	60.8 \pm 11.3	30.5 \pm 4.3	2.03	0.02*
Absolute PB blasts ($\times 10^9/\text{l}$)	4.6 \pm 1.2	17.8 \pm 10.5	0.96	1.23
BM blasts (%)	62.3 \pm 5.1	66.6 \pm 5.4	0.53	0.57
<i>CD34 n</i>				
5–	3 (17.6)	2 (15.4)	0.869	0.633
25+	14 (82.4)	11 (84.6)		
<i>HLA-DR n</i>				
4–	1 (5.9)	1 (7.7)	0.844	0.68
26+	18 (94.1)	12 (92.3)		
<i>CD117 n</i>				
4–	1 (5.9)	3 (23.1)	1.88	0.29
26+	16 (94.1)	10 (76.9)		
<i>FAB n</i>				
5 M0	1 (7.7)	4 (23.5)		
7 M1	4 (30.8)	3 (17.7)		
8 M2	4 (30.8)	4 (23.5)		
2 M3	1 (7.7)	1 (5.9)		
5 M4	–	5 (29.4)		
2 M5	2 (15.3)	–	12.6	0.04*
1 M6	1 (7.7)	–		
TPS levels ($\times 10^{-2}$)	–	7.53 \pm 1.79		

TLC, total leukocytic counts; PB, peripheral blood; BM, bone marrow; FAB, French–American–British Classification; M, myeloid.

* Significance level: $p < 0.05$.**Table 2** Comparative study of TPS mRNA transcripts levels in different FAB subtypes in patients group.

	TPS gene expression levels ($\times 10^{-2}$)	<i>t</i> -Test	<i>p</i>
M4	12.1 \pm 3.4		
M0, 1, 2, 3	4.6 \pm 1.3	2.39	0.03*

FAB, French–American–British Classification; M, myeloid.

* Significance level: $p < 0.05$.**Table 3** Correlation between TPS levels and other prognostic factors in patients group.

Parameters	TPS gene expression levels		
	<i>r</i>	<i>p</i>	S
Age (years)	0.17	0.37	NS
TLC ($\times 10^9/\text{L}$)	0.48	0.007*	HS
Hemoglobin (g/dl)	–0.286	1.25	NS
Platelets ($\times 10^3/\text{ml}$)	–0.231	0.22	NS
Absolute PB blasts ($\times 10^9/\text{l}$)	0.427	0.01*	S
BM blasts (%)	–0.204	0.28	NS
CD34 (%)	0.485	0.007*	HS
HLA-DR (%)	0.328	0.07	NS
CD117 (%)	0.414	0.02*	S

TLC, total leukocytic counts; PB, peripheral blood; BM, bone marrow; S, significant; HS, highly significant; NS, non-significant.

* Significance level: $p < 0.05$.**Table 4** TPS gene expression among the studied group and patients' outcome during the follow up period.

Fate of patients	TPS gene expression			
	Negative cases (13)		Positive cases (17)	
	No.	%	No.	%
CR	11	84.6	8	47.1
Relapse/Death	2	15.4	9	52.9
X ² test	4.47			
<i>p</i> value	0.04*			

CR, complete remission.

* Significance level: $p < 0.05$.

used (immunohistochemistry or Northern blot analysis). In the current study, TPS expression showed a statistical significant association with FAB subtypes; it was expressed among all M4 patients, 80% of M0; 42.9% of M1; 50% of M2 subtype and in one patient with M3 similar results were reported previously [3], and added that TPS expression in M4 is indicative of minimal differentiation along the mast cell pathway but the maturation arrest did not allow for terminal differentiation and maturation [5]. Furthermore, we measured TPS mRNA levels and showed statistically significant higher values in patients with M4 compared to other FAB subtypes. Similar results were obtained by a previous report in which TPS levels were detected in serum [8].

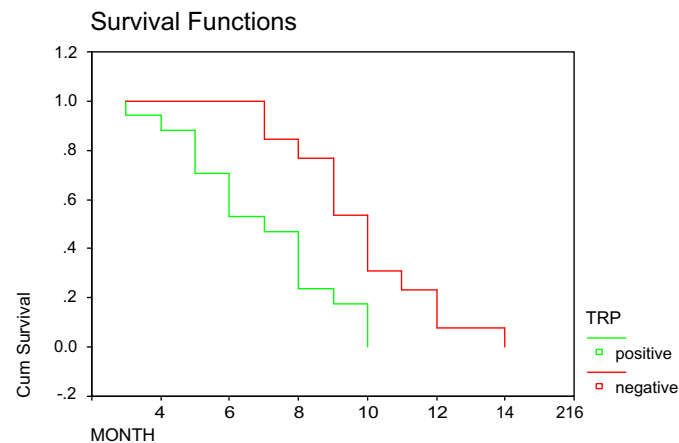


Figure 2 Survival analysis for ANLL patients using Kaplan–Meier curve.

In this report, patients with positive TPS showed higher TLC, lower hemoglobin levels and platelet counts compared to negative cases with a statistical significant difference. Similar results were also reported previously [13]. Again, we elucidated the value of TPS expression in relation to other prognostic factors. TPS expression was directly correlated with total leukocytic counts, absolute blast counts in peripheral blood, suggesting its correlation with disease severity and tumor burden. Similarly, overexpression of TPS was previously correlated with poor survival in ANLL [9]. Moreover, we demonstrated that TPS expression was directly correlated with CD34 and CD117 levels. Previous studies showed that these ANLL blasts frequently co-express tryptase with additional mast cell lineage-and/or basophil-related differentiation antigens including CD117 [8].

In this report, after induction chemotherapy TPS levels were decreased in the eight patients who achieved complete remission during the follow up period. The rest of the positive cases (nine patients) revealed statistically higher TPS mRNA values; five showed blast cell persistence by bone marrow examination, and recurred shortly after, during follow up period. The other four patients showed persistent elevated TPS expression despite hematologic complete remission, these patients showed disease recurrence during consolidation treatment. The regrowth of blasts was associated with significant increase in TPS levels. Similar results were obtained by Sperr et al. [9] who found that the serum TPS levels were initially high, decreased with treatment and were re-elevated with the onset of relapse and that hematologic or/and molecular relapse is accompanied or even preceded by a recurrent increase in TPS [13]. Moreover, previous data showed that persistently elevated TPS in hematologic CR is indicative of minimal residual ANLL and is a poor prognostic marker with high risk of relapse [4], which may significantly influence the treatment plan in these patients [14]. Others explained the slow decrease in TPS even after a long latent period despite continuous hematologic CR indicating the time taken until residual ANLL cells disappear [15,16]. The risk of relapse in patients with positive TPS expression was six times more frequent than those with negative expression with shorter overall survival as previously reported [9]. On the contrary, the CR rate was found previously to be similar in patients with

normal serum TPS compared with those with enhanced enzyme levels [5].

In conclusion, TPS is considered as a useful novel marker for diagnosis and prognosis of myeloid neoplasia. The availability of such marker for monitoring minimal residual disease in hematologic complete remission is of major clinical importance and is helpful in planning treatment in individual patients with ANLL. Furthermore, anti-TPS could be used as a suitable target for immunotherapeutic intervention.

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